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## Catabolic Pathways and Role of Ligninases for the Degradation of Lignin Substructure Models by White-Rot Fungi

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This paper summarizes our research on lignin biodegradation at Wood Research Institute, Kyoto University. The purpose of the investigation is to elucidate the degradation mechanism of lignin by microorganisms and enzymes, and to gain the background knowledge needed to use lignin degrading microorganisms and their enzymes in biochemical processing of woods.

Lignin is a three dimensional anomalous polymer containing many different stable carbon-to-carbon and ether linkages between monomeric phenylpropane units (Fig. 1). Lignin is a complex natural plastic and generally distributed as a matrix component in the spaces of intercellulose microfibrils in primary and secondary walls, and in middle lamellae, and functions to connect cells one another to harden the cell walls of woods to give a laminated composite material.

Hence development of economically and environmentally suitable delignification processes has been a major problem in paper mill industries and chemical processing of woods. Microbial delignification is still premature to be used in these industrial processes, but several potential applications such as bleaching chemical pulps, improving mechanical pulps, waste water treatments etc. would be developed in near future.

Lignin is a so complicated polymer and it is difficult to elucidate the degradation mechanism by analyses of degradation products of lignin, and therefore, lignin substructure model compounds have largely contributed for the elucidation of catabolic pathways of lignin by microorganisms<sup>1)</sup>.

Thus, we<sup>2)</sup> synthesized several oligolignols and used them for the elucidation of the mechanism of lignin biodegradation with soil isolate, *Fusarium solani* M-13-1

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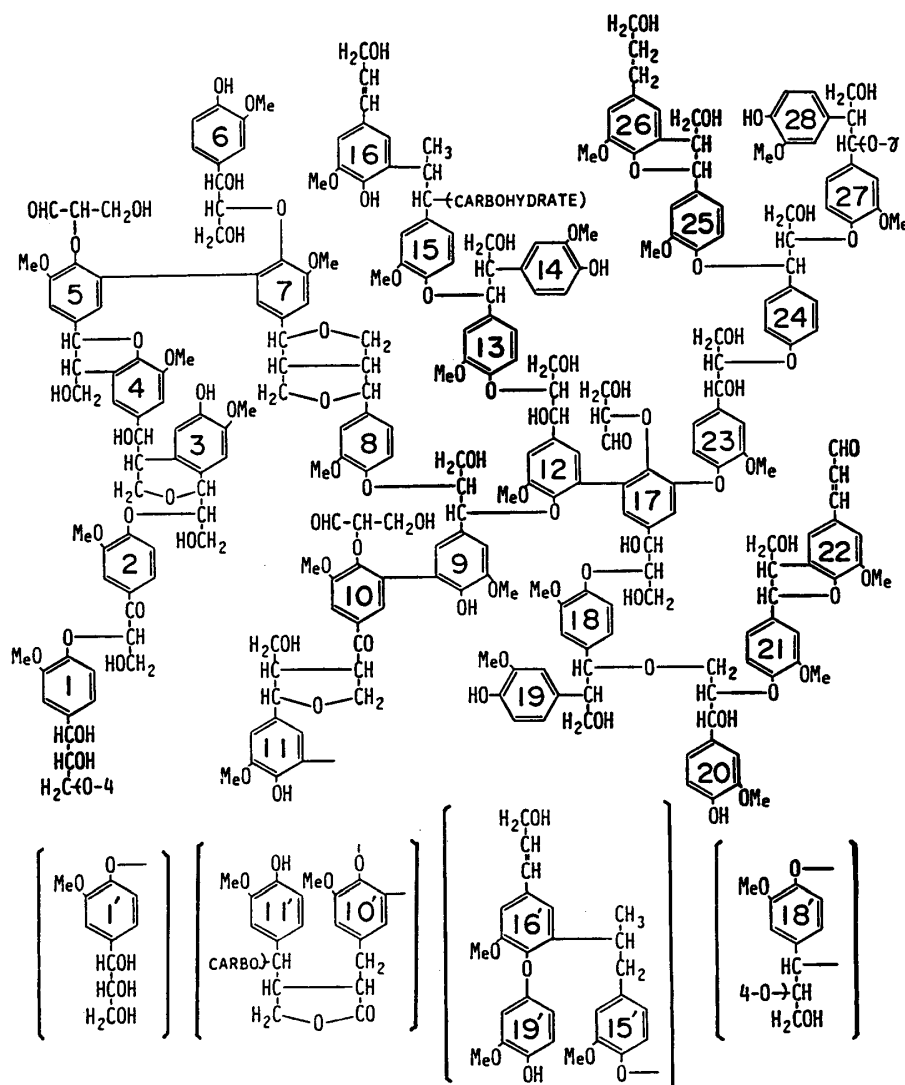


Fig. 1. A structural model of softwood lignin illustrating the major types of linkages between the guaiacylpropane units (A. Sakakibara, Wood Sci. Technol., 14, 89 (1980)).

using coniferyl alcohol DHP as the sole carbon source<sup>3)</sup>, and with *Phanerochaete chrysosporium* and *Coriolus versicolor*, typical white-rot fungi, respectively.

Oligolignols were incubated with shaking culture of *F. solani* M-13-1, with ligninolytic cultures<sup>4)</sup> of *P. chrysosporium* and *C. versicolor*, and with laccase from *C. versicolor* and lignin peroxidase from *P. chrysosporium*. The degradation products were isolated and identified by NMR and mass spectrometry to establish the catabolic pathways for the oligolignols. This paper deals with the investigations with the white-rot fungi.

# 1. Degradation of phenolic dilignols by culture of *P. chrysosporium*

*Phanerochaete chrysosporium* and *C. versicolor* degrade both phenolic and nonphenolic  $\beta$ -O-4 compounds. A phenolic  $\beta$ -O-4 model, arylglycerol- $\beta$ -coniferyl ether (1) is converted by *Phanerochaete* (pathway B, Fig. 2) to arylglycerol- $\beta$ -guaiacylglycerol ether<sup>5</sup> (2') which is converted to vanillic acid ether (5) via vanillin ether (4). The vanillic acid ether is then degraded either to glycerol-2-vanillic acid ether (6) and methoxy-p-benzoquinone (7') by alkyl-phenyl cleavage or to vanillyl alcohol (9), probably via vanillin as primary product by oxidative  $C_\alpha$ - $C_\beta$  cleavage, as found for nonphenolic  $\beta$ -O-4 models<sup>1</sup>. Both alkyl-phenyl and  $C_\alpha$ - $C_\beta$  cleavage could occur via phenoxy radicals of the substrate mediated by lignin peroxidase as discussed later in the degradation of  $\beta$ -1 compounds by laccase.

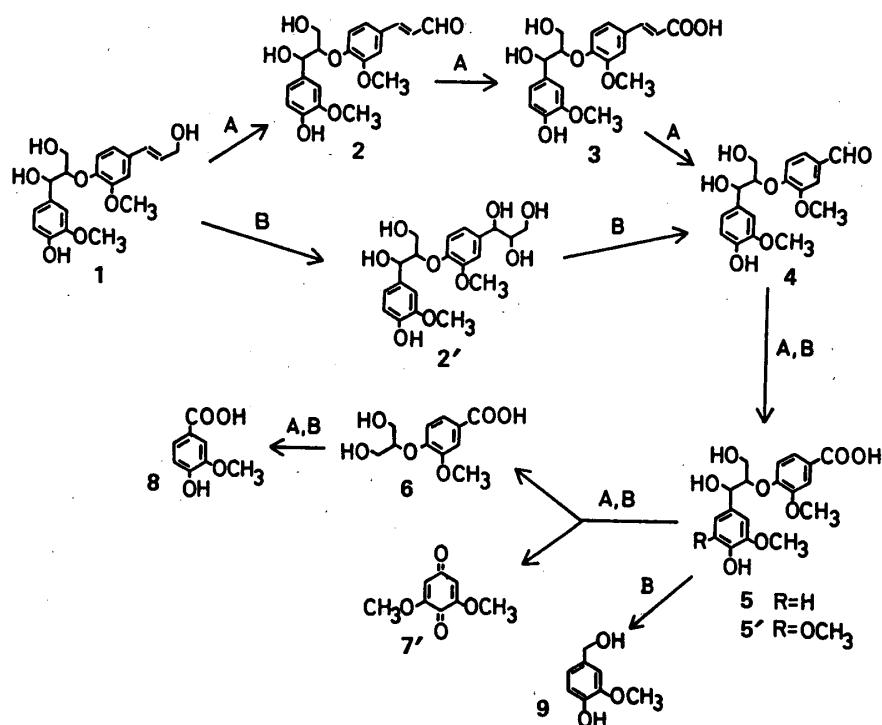


Fig. 2. Degradation pathways of guaiacylglycerol- $\beta$ -coniferyl ether by *F. solani* M-13-1 and *Phanerochaete chrysosporium*. A, Pathway by *Fusarium*; B, pathway by *Phanerochaete*.

The pathway B in Fig. 3 shows the degradation pathways of dehydrodiconiferyl alcohol by *P. chrysosporium*<sup>1,6</sup>. Dehydrodiconiferyl alcohol (10), which is a major lignin substructure, is degraded by *P. chrysosporium* to phenylcoumaran  $\alpha'$ -aldehyde (13) via glycerol derivative. Then, phenylcoumaran  $\alpha'$ -aldehyde is partly converted either to phenylcoumarone (14) or to a propiosyringone derivative (15). The latter is converted to 5-carboxyvanillic acid (16) and syringic acid (17), or to 5-

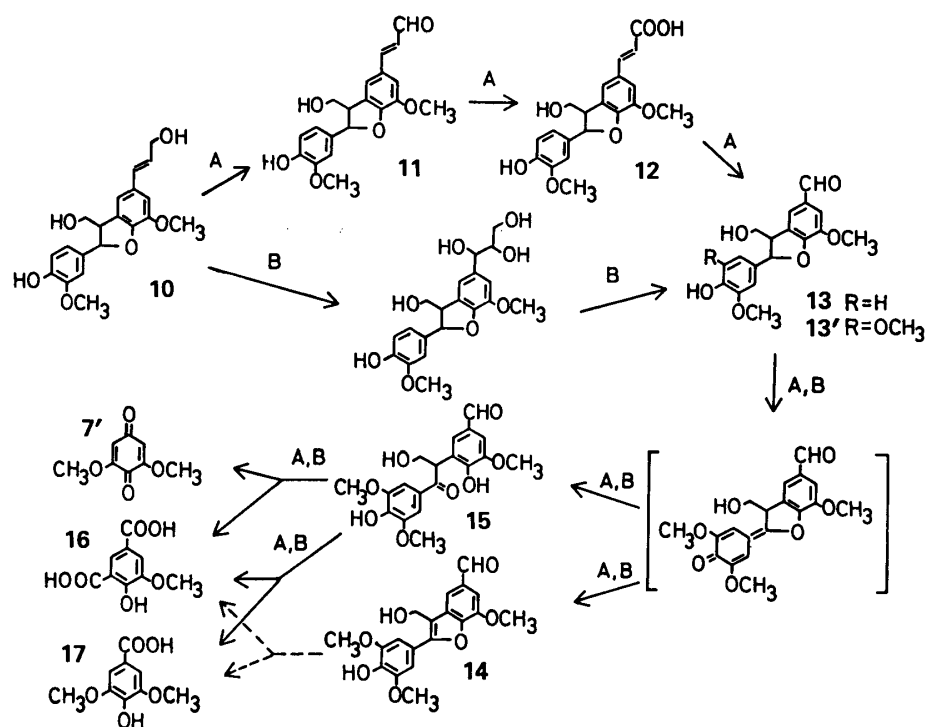


Fig. 3. Degradation pathways of dehydrodiconiferyl alcohol by *F. solani* M-13-1 and *P. chrysosporium*. A, Pathway by *Fusarium*; B, pathway by *Phanerochaete*.

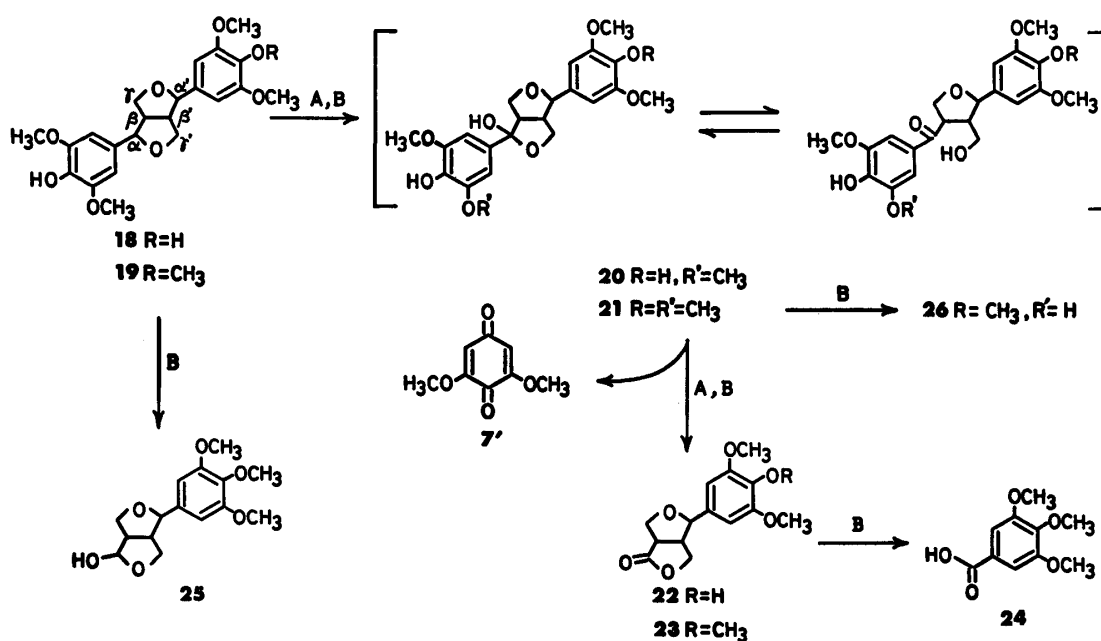


Fig. 4. Degradation pathways of syringaresinol derivatives by *F. solani* M-13-1 and *P. chrysosporium*. A, Pathway by *Fusarium*; B, pathway by *Phanerochaete*.

carboxyvanillic acid and 2,6-dimethoxy-p-benzoquinone (7'), respectively by *Phanerochaete*. The results indicate that degradation of phenolic phenylcoumaran by the fungus proceeds either via alkyl-phenyl cleavage or via C<sub>α</sub>-C<sub>β</sub> cleavage probably mediated by lignin peroxidase.

Fig. 4 (pathway B) shows the degradation pathways of syringaresinol derivatives by *P. chrysosporium*<sup>1,7)</sup>. With *P. chrysosporium* syringaresinol (18), a major substructure of hardwood lignin, and its monomethyl ether (19) are converted to the C<sub>α</sub>-oxidized compounds (20, 21), 3-O-demethylated compound (26), γ-lactons (22, 23), methoxy-p-benzoquinone (7') and lactol (25). γ-Lacton was converted to 3,4,5-trimethoxybenzoic acid (24) via C<sub>α</sub>-C<sub>β</sub> cleavage. The formation of these degradation products indicates that the resinols were degraded via oxidative alkyl-phenyl cleavage and C<sub>α</sub>-C<sub>β</sub> cleavage, both probably mediated by lignin peroxidase. d, l-Pinoresinol monomethyl ether is converted analogous degradation products. The C<sub>α</sub>-oxidized compounds (20, 21) occur in two chemical structures, hemiketal-keto alcohol, due to the ring-chain tautomerism.

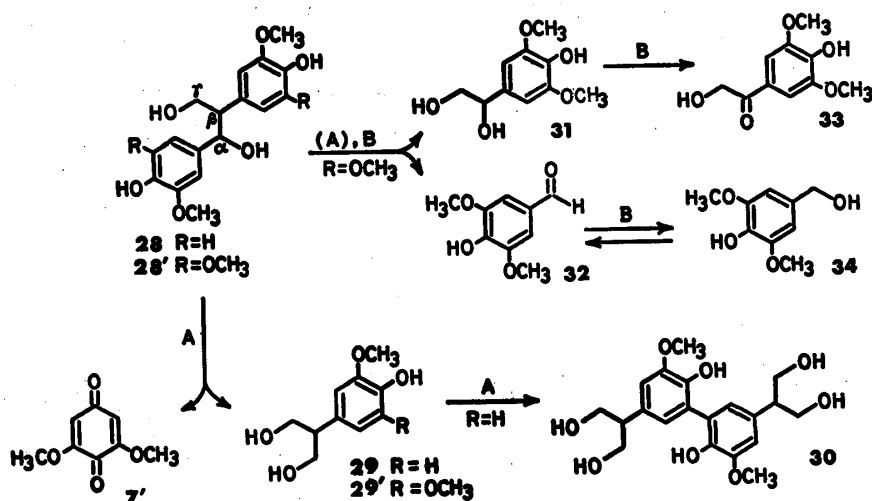


Fig. 5. Degradation pathways of 1,2-diarylpropane-1,3-diols by *F. solani* M-13-1 and *P. chrysosporium*. A, Pathway by *Fusarium*; B, pathway by *Phanerochaete*.

Fig. 5 (pathway B) shows the degradation pathways of 1,2-disyringylpropane-1,3-diol (28') one of the major substructure of lignin by ligninolytic culture of *P. chrysosporium*<sup>1,8)</sup>. The compound was degraded to syringylglycol (31), α-hydroxyacetosyringone (33), syringaldehyde (32) and syringyl alcohol (34), but 2,6-dimethoxy-p-benzoquinone (7') was scarcely detected. This suggests that the degradation of phenolic β-1 models with *P. chrysosporium* proceeds mainly via C<sub>α</sub>-C<sub>β</sub> cleavage.

## 2. Degradation of nonphenolic dilignols by cultures of *P. chrysosporium* and *C. versicolor*

Arylglycerol- $\beta$ -aryl ether bond is the most frequent interphenylpropane linkage which roles to connect main lignin substructures to give a lignin macromolecule. It is, therefore, very important to elucidate the degradation mechanism for non-phenolic  $\beta$ -O-4 substructure by lignin degrading fungi in relation to the depolymerization of lignin by fungi. When we started degradation study in 1981 on  $\beta$ -O-4 model compounds (35) using *P. chrysosporium* arylglycerol (36), guaiacol (37),  $C_6C_\alpha$  (benzyl alcohol) derivative (38) and guaiacoxyethanol (39) had been identified as degradation products of nonphenolic arylglycerol- $\beta$ -guaiacyl ether by *P. chrysosporium*. Guaiacoxyethanol (39) was suggested to be formed by retroaldol reaction<sup>12)</sup> of  $\gamma$ -aldehydic  $\beta$ -O-4 dimers shown in Fig. 6. However, no evidence had been obtained on the mechanism of  $C_\alpha$ - $C_\beta$  cleavage, and formation of arylglycerol and guaiacoxyethanol. So, to elucidate the mechanism of  $C_\alpha$ - $C_\beta$  cleavage we synthesized a trimer (40) composed of  $\beta$ -O-4 and  $\alpha$ -O- $\gamma$  substructure, i.e.  $\gamma$ -benzyl ether of  $\beta$ -O-4 substructure model, and used it as substrate for ligninolytic culture of *P. chrysosporium*<sup>9)</sup>. Fig. 7 shows the degradation of  $\gamma$ -benzyl ether of  $\beta$ -O-4 model (40) with *Phanerochaete*. Benzyloxyethanol (41), a  $C_\beta$ - $C_\gamma$  fragment compound was first identified by our investigation, in addition to a  $C_6C_\alpha$  derivative (38) and arylglycerol derivative (42) as degradation products. Thus, we, lignin biochemists now understand that the arylglycerol moiety of  $\beta$ -O-4 model compounds is cleaved between  $C_\alpha$  and  $C_\beta$  in side chain to give a  $C_6C_\alpha$  derivative and glycol ( $C_\beta$ - $C_\gamma$  fragment compound)<sup>1)</sup>. Afterwards, the  $C_\alpha$ - $C_\beta$  cleavage of  $\beta$ -O-4 model compound in accordance with our result by an extracellular enzyme which was later characterized as lignin peroxidase, was demonstrated by Kirk's group<sup>10)</sup> and Gold's group<sup>11)</sup>, respectively.

As for the formation of guaiacoxyethanol which was previously suggested to be formed via retroaldol reaction from  $\gamma$ -aldehydic  $\beta$ -O-4 dimers (Fig. 6) by Gold's

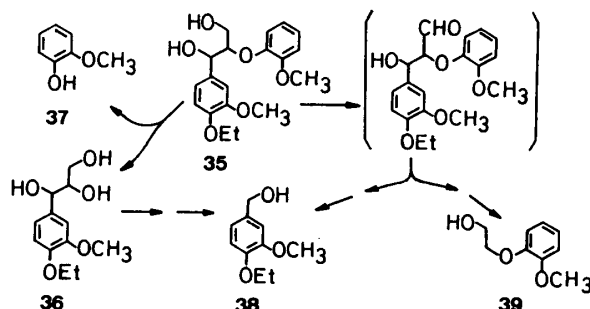


Fig. 6. Degradation pathways of nonphenolic arylglycerol- $\beta$ -aryl ether models by *P. chrysosporium*.

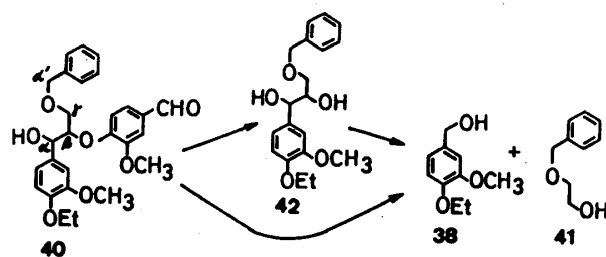


Fig. 7. Degradation pathways of a trimer composed of  $\beta$ -O-4 and  $\alpha$ -O- $\gamma$  substructure,  $\gamma$ -benzyl ether of  $\beta$ -O-4 substructure model by *P. chrysosporium*.

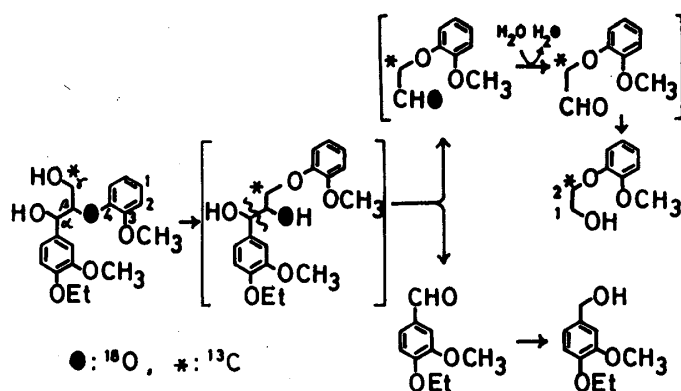


Fig. 8. Formation mechanism of guaiacoxyethanol from 4-ethoxy-3-methoxyphenylglycerol- $\beta$ -guaiacyl ether by *P. chrysosporium*.

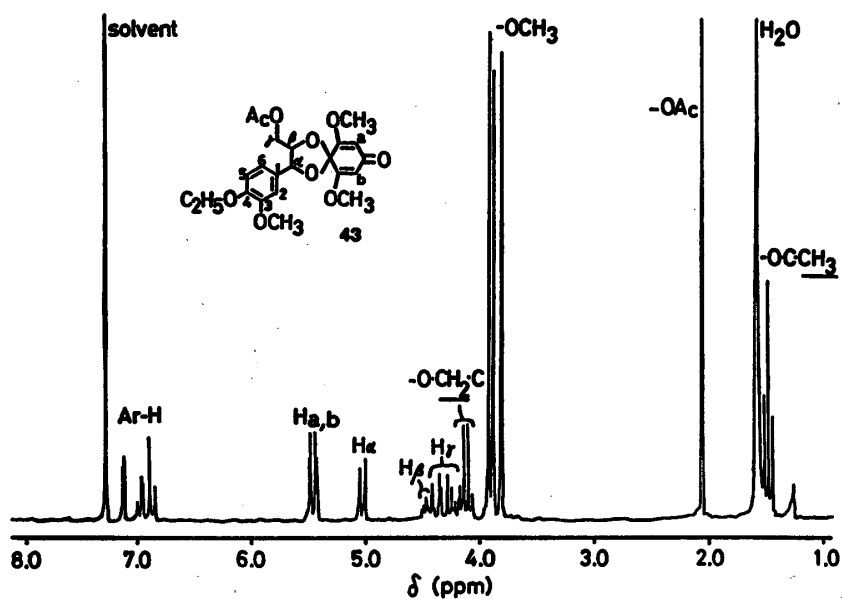


Fig. 9. NMR spectrum of p-benzoquinone monoketal formed from 4-ethoxy-3-methoxyphenylglycerol- $\beta$ -syringaldehyde ether by *Coriolus versicolor*.



group<sup>12)</sup>, we found by isotopic investigation with arylglycerol- $\beta$ - $^{18}\text{O}$ -guaiacyl ether and arylglycerol-( $\gamma$ - $^{13}\text{C}$ )- $\beta$ -guaiacyl ether as substrate for ligninolytic culture of *P. chrysosporium* that the guaiacyl group at the  $\beta$ -position of the substrate is rearranged to the adjacent  $\gamma$ -position, as illustrated in Fig. 8 and then followed by  $\text{C}_\alpha$ - $\text{C}_\beta$  cleavage to give guaiacoxyethanol<sup>13)</sup>. Thus, it is unlikely that guaiacoxyethanol was formed via retroaldol reaction of  $\gamma$ -aldehydic  $\beta$ -O-4 dimers. Gold's group recently confirmed<sup>14)</sup> our results by isotopic investigations and withdrew their previous proposal.

Very recently we<sup>15)</sup> identified a new product, p-benzoquinone monoketal (43) as a catabolite of 4-ethoxy-3-methoxyphenylglycerol- $\beta$ -syringaldehyde ether by ligninolytic culture of *C. versicolor*. Fig. 9 shows the NMR spectrum of the compound, and Fig. 10 shows mass spectrum of the compound. This compound was also produced in the degradation of 4-ethoxy-3-methoxyphenylglycerol- $\beta$ -syringic acid ether by lignin peroxidase as discussed later.

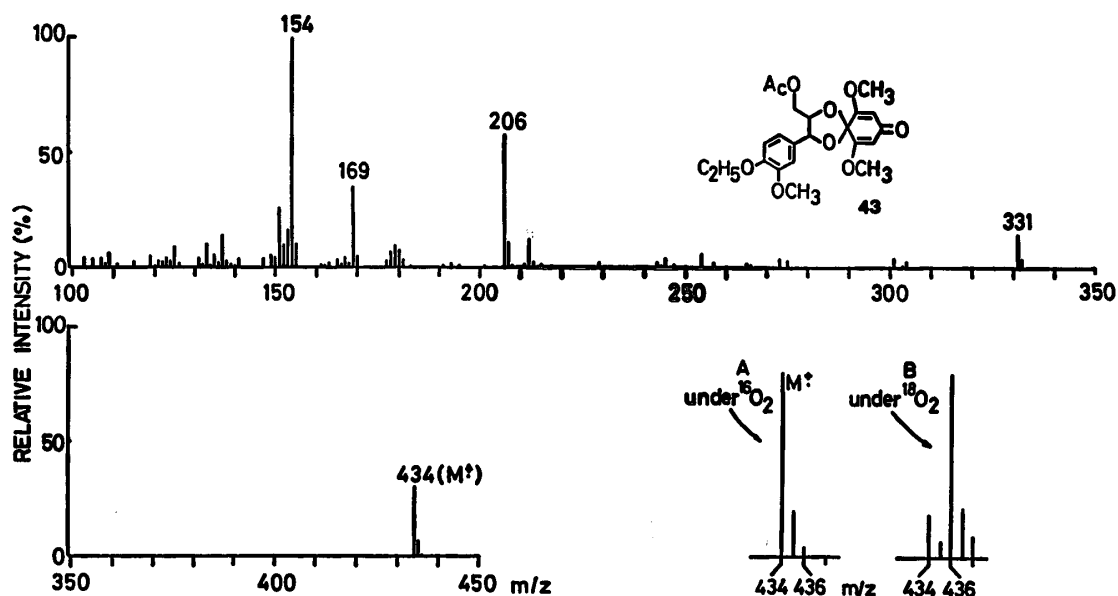


Fig. 10. Mass spectrum of p-benzoquinone monoketal formed from 4-ethoxy-3-methoxyphenylglycerol- $\beta$ -syringaldehyde ether by *Coriolus versicolor*. A, Experiment under  $^{16}\text{O}_2$ ; B, experiment under  $^{18}\text{O}_2$ .  $\text{M}^+$  is 436 which indicates the incorporation of  $^{18}\text{O}$  into the compound (cf. Fig. 20).

As for the formation of arylglycerol from  $\beta$ -O-4 compounds earlier papers suggested that hydrolysis<sup>16)</sup> and/or  $\beta$ -hydroxylation<sup>17)</sup> of arylglycerol- $\beta$ -aryl ether occurs. However, as shown in Fig. 11, we<sup>9,18)</sup> found that the isolated arylglycerol in the degradation of  $\text{C}_\alpha$  and  $\text{C}_\beta$  double deuterated  $\beta$ -O-4 model compounds retained deuterium almost 100% at both  $\text{C}_\alpha$  and  $\text{C}_\beta$  positions. We<sup>19,20)</sup> also found that  $^{18}\text{O}$  was not incorporated into  $\text{C}_\beta$  but partly into  $\text{C}_\alpha$  of arylglycerol from  $\text{H}_2^{18}\text{O}$  in the degradation

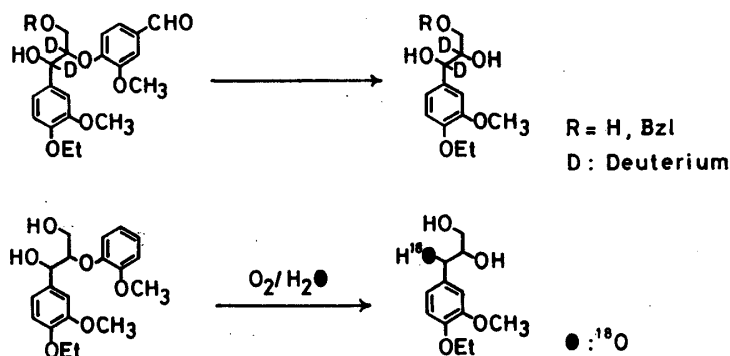


Fig. 11. Formation of arylglycerol from  $\beta$ -O-4 model compound by *P. chrysosporium*.

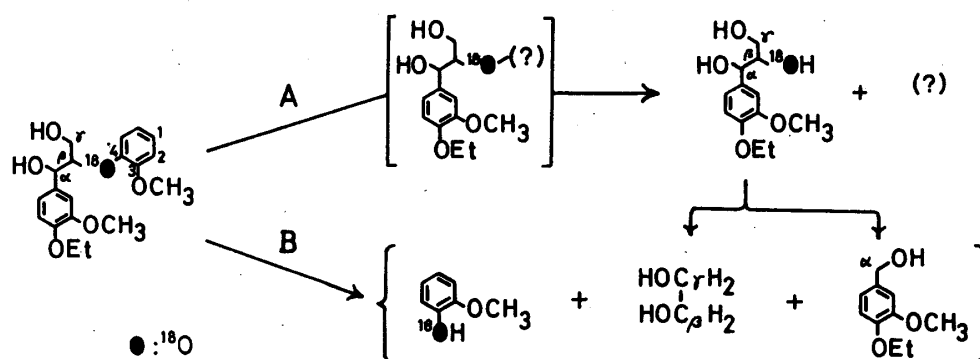


Fig. 12. Degradation of arylglycerol- $\beta$ - $^{18}\text{O}$ -guaiacyl ether by *P. chrysosporium*.

of  $\beta$ -O-4 model compound under  $\text{H}_2^{18}\text{O}$ . These results indicate neither  $\text{C}_\beta$  hydroxylation nor hydrolysis of  $\beta$ -O-4 compounds occurs in the formation of arylglycerol. Our further experiment<sup>21)</sup> with arylglycerol- $\beta$ - $^{18}\text{O}$ -guaiacyl ether as substrate for ligninolytic culture of *P. chrysosporium* (Fig. 12) showed that  $^{18}\text{O}$  was retained almost 100% at  $\text{C}_\beta$  of the arylglycerol and hydroxy group of the guaiacol as degradation product, respectively. The guaiacol- $^{18}\text{O}$  could be derived from a hemiketal intermediate in  $\text{C}_\alpha$ - $\text{C}_\beta$  cleavage mediated by lignin peroxidase. For the formation of arylglycerol- $\beta$ - $^{18}\text{O}$  we supposed  $\beta$ -aromatic ring cleavage product as a precursor, and have searched for  $\beta$ -aromatic ring cleavage products in the ligninolytic culture of *P. chrysosporium* with arylglycerol- $\beta$ -guaiacyl ether.

As shown in Fig. 13, we<sup>22)</sup> identified  $\beta$ ,  $\gamma$ -cyclic carbonate of arylglycerol (44) as the first aromatic ring cleavage product. Isotope experiment with 4-ethoxy-3-methoxyphenylglycerol- $\beta$ -guaiacyl- $\text{U-}^{13}\text{C}$  ether showed that the carbonate carbon was derived from guaiacyl ring carbon, indicating that the ring cleavage of  $\beta$ -etherated guaiacol occurred. The same result was obtained in the experiment with ligninolytic culture of *C. versicolor*<sup>23)</sup>.

In further experiments, we<sup>23)</sup> found that the ligninolytic culture of *P. chrysosporium*

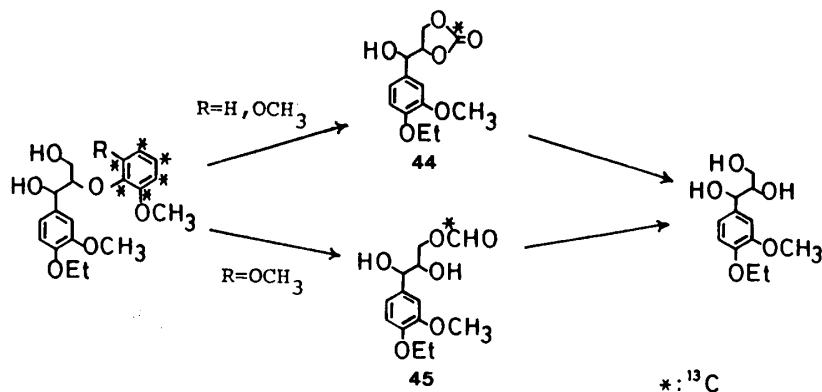


Fig. 13. Formation of  $\beta, \gamma$ -cyclic carbonate,  $\gamma$ -formate of arylglycerol from 4-ethoxy-3-methoxyphenylglycerol- $\beta$ -guaiacyl (syringyl)- $\text{U-}^{13}\text{C}$  ether by *P. chrysosporium* and *C. versicolor*.

and *C. versicolor* degraded 4-ethoxy-3-methoxyphenylglycerol- $\beta$ -syringyl ether to give 4-ethoxy-3-methoxyphenylglycerol- $\beta$ -formate ester (45), and that the formate carbon was derived from syringyl ring by using 4-ethoxy-3-methoxyphenylglycerol- $\beta$ -syringyl- $\text{U-}^{13}\text{C}$  ether as substrate. These results indicated that some of the arylglycerol is

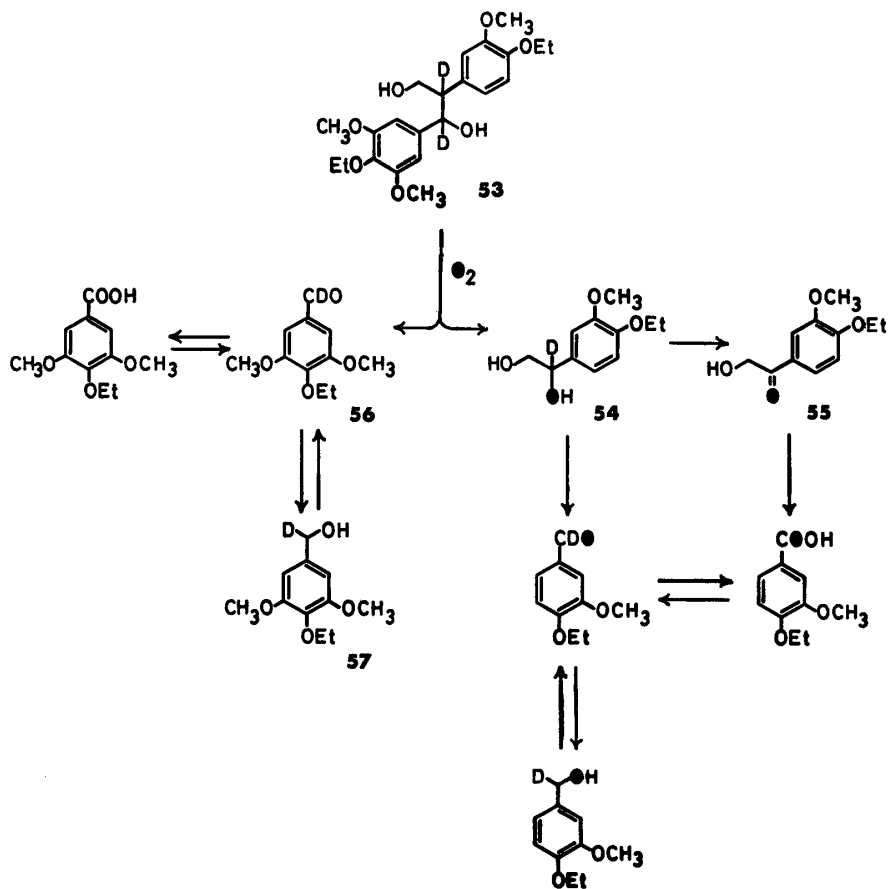


Fig. 14. Degradation pathways of nonphenolic 1,2-diarylpropane-1,3-diols by *P. chrysosporium*. D, Deuterium; ●,  $^{18}\text{O}$ .

formed by the cleavage of aromatic ring of  $\beta$ -etherated aryl group.

Fig. 14 shows the degradation pathways of the nonphenolic diarylpropane model compound (53). The compound was found to be degraded to three major product, phenylglycol (54),  $\alpha$ -hydroxyacetophenone (55) and benzaldehyde (56) by Nakatsubo et al.<sup>24)</sup>, and Gold's group<sup>25)</sup>, respectively. Kirk and Nakatsubo<sup>26)</sup> further showed, by mass spectrometric analysis of the phenylglycol and the aromatic alcohol formed from deuterated  $\beta$ -1 substrate under  $^{18}\text{O}_2$  atmosphere, that hydrogen atoms at  $\text{C}_\alpha$  and  $\text{C}_\beta$  are not lost, and that the benzyl hydroxyl oxygen atom of the phenylglycol (54) is derived from molecular oxygen. We<sup>19)</sup> confirmed these results with ligninolytic culture of *P. chrysosporium*, and also found that the ligninolytic culture of *C. versicolor* degrades nonphenolic diarylpropane-1,3-diol via the same pathway<sup>27)</sup>. Afterwards, Tien and Kirk<sup>10,28)</sup> and Gold's group<sup>11,29)</sup> established that  $\beta$ -1 compounds are cleaved to benzaldehyde and phenylglycol by the extracellular  $\text{H}_2\text{O}_2$ -dependent enzyme, now called lignin peroxidase, from *P. chrysosporium*.

*Phanerochaete chrysosporium* degraded 4-O-methyldehydrodiconiferyl alcohol to a vanillic acid derivative via glycerol derivative. The coumaran ring is then partly converted to coumarone, which is degraded slowly to a  $\text{C}_6\text{-C}_1$  acid<sup>30)</sup>. However, the mechanism of  $\text{C}_\alpha\text{-C}_\beta$  cleavage of nonphenolic phenylcoumaran has not yet been established.

Syringaresinol dimethyl ether is hardly degraded by *P. chrysosporium*. No  $\text{C}_\alpha\text{-C}_\beta$  cleavage products such as 3,4,5-trimethoxybenzoic acid and the corresponding derivatives could be detected<sup>7)</sup>.

### 3. Degradation of monomeric aromatic compounds by *P. chrysosporium*

It was found that noncondensed guaiacyl model compounds were converted to vanillic acid and methoxyhydroquinone by *P. chrysosporium* by Eriksson's group<sup>31)</sup>, whereas condensed guaiacyl substructure models (60) and syringyl models were degraded to 5-carboxyvanillic acid (61) and to syringic acid (17) and 2,6-dimethoxy-p-benzoquinone (7'), respectively, by *P. chrysosporium*<sup>1,32)</sup> (Fig. 15). We found that dehydrodivanillic acid (60) is converted to 5-carboxyvanillic acid (61), which is then converted to 5-hydroxyvanillic acid (62) and/or 5-carboxyprotocatechuic acid (63) by ligninolytic culture of *P. chrysosporium*. The latter two compounds are converted to gallic acid (64). On the other hand, syringic acid (17) is 3-O-demethylated to 5-hydroxyvanillic acid (62), which is metabolized via gallic acid (64) by *P. chrysosporium*. The results strongly suggest that phenylcoumaran and biphenyl substructures of lignin are partly degraded to 5-carboxyvanillic acid, and syringyl moieties of lignin to syringic acid. Both are converted to gallic acid as the final aromatic monomer.

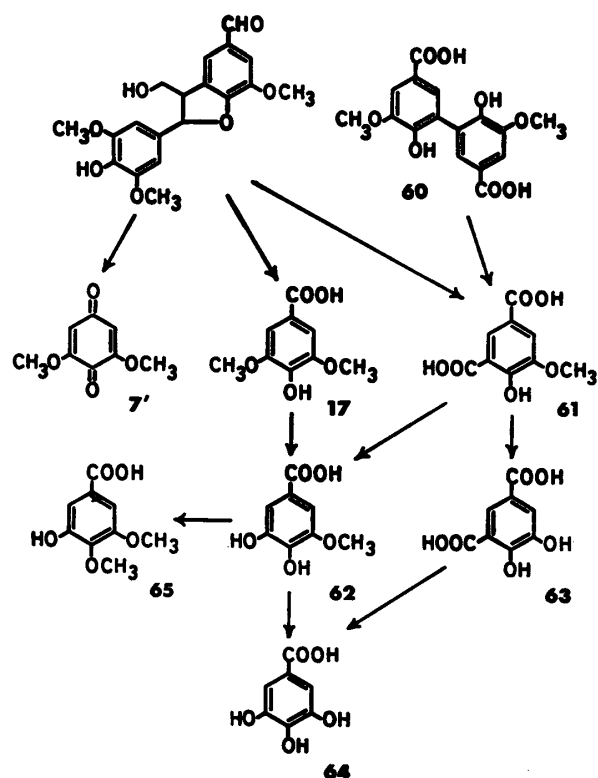


Fig. 15. Degradative transformation of syringic acid and 5-carboxyvanillic acid by *P. chrysosporium*.

#### 4. Degradation of diarylpropane-1,3-diol models by laccase

We used electrophoretically homogeneous laccase of *C. versicolor* for this investigation. We synthesized 1,2-bis(4-hydroxy-3,5-dimethoxyphenyl)propane-1,3-diol, its diethyl ether and two monoethyl ethers as substrate<sup>33)</sup>. Diethyl ether of 1,2-bis(4-hydroxy-3,5-dimethoxyphenyl)propane-1,3-diol which has no phenolic hydroxyl group was not oxidized by the laccase as expected. Here, the degradation pathways of the two monoethyl ethers by the laccase are discussed. Fig. 16 shows the degradation pathways of 1-(4-hydroxy-3,5-dimethoxyphenyl)-2-(4-ethoxy-3,5-dimethoxyphenyl)propane-1,3-diol [3] by the laccase. The substrate was degraded to yield 1-(4-hydroxy-3,5-dimethoxyphenyl)-2-(4-ethoxy-3,5-dimethoxyphenyl)propane-1-one-3-ol [9], 2-(4-ethoxy-3,5-dimethoxyphenyl)-3-hydroxypropanal [10], 2,6-dimethoxyhydroquinone [11], syringaldehyde [7] and 1-(4-ethoxy-3,5-dimethoxyphenyl)-2-hydroxyethanone [12]. 2,6-Dimethoxyhydroquinone could be oxidized to 2,6-dimethoxy-p-benzoquinone [8].

Isotope experiment<sup>33)</sup> with  $^{18}\text{O}_2$  and  $\text{H}_2^{18}\text{O}$  showed that  $^{18}\text{O}_2$  was incorporated into  $\alpha$ -carbonyl oxygen of the ethanone, and that oxygen of hydroxyl group of the hydroquinone and benzoquinone was derived from  $\text{H}_2^{18}\text{O}$ . Syringaldehyde and the ethanone could be formed by  $\text{C}_\alpha\text{-C}_\beta$  cleavage of the phenoxy radicals of the sub-

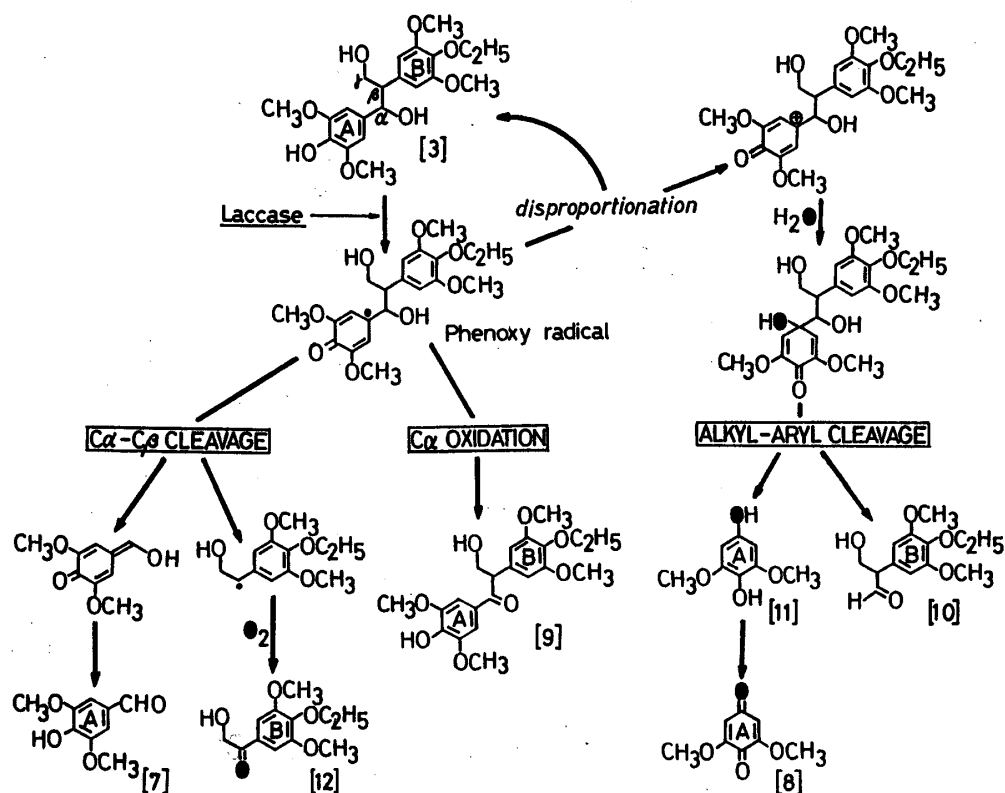


Fig. 16. Degradation pathways of 1-(4-hydroxy-3,5-dimethoxyphenyl)-2-(4-ethoxy-3,5-dimethoxyphenyl)propane-1,3-diol by laccase.  
●,  $^{18}\text{O}$ .

strate, and the hydroxypropanal and the hydroquinone could be formed by alkyl-phenyl cleavage by disproportionation of the phenoxyl radicals of the substrate to the corresponding cation and the subsequent reaction of the cation with  $\text{H}_2\text{O}$ . These results are in good accordance with the pathways in Fig. 16.

Fig. 17 shows the degradation pathways of 1-(4-ethoxy-3,5-dimethoxyphenyl)-2-(4-hydroxy-3,5-dimethoxyphenyl)propane-1,3-diol [4] by the laccase<sup>33</sup>. The substrate was degraded to yield 4-O-ethylsyringaldehyde [13] and syringylglycol [5] which could be converted to  $\alpha$ -hydroxyacetosyringone [6]. Isotope experiments with  $^{18}\text{O}_2$  and  $\text{H}_2^{18}\text{O}$  showed that oxygen of  $\alpha$ -hydroxyl group of syringylglycol and carbonyl group of acetosyringone was derived from  $\text{H}_2^{18}\text{O}$ . The structures of the products and isotope experiments indicated that the substrate was degraded via the pathways shown in Fig. 17. The results showed that  $\text{C}_\alpha\text{-C}_\beta$  cleavage as well as alkyl-phenyl cleavage and  $\text{C}_\alpha$  oxidation are generally involved in the degradation of  $\beta$ -1 compound catalyzed by the laccase as in the degradation of nonphenolic  $\beta$ -1 substructure model compounds by lignin peroxidase.

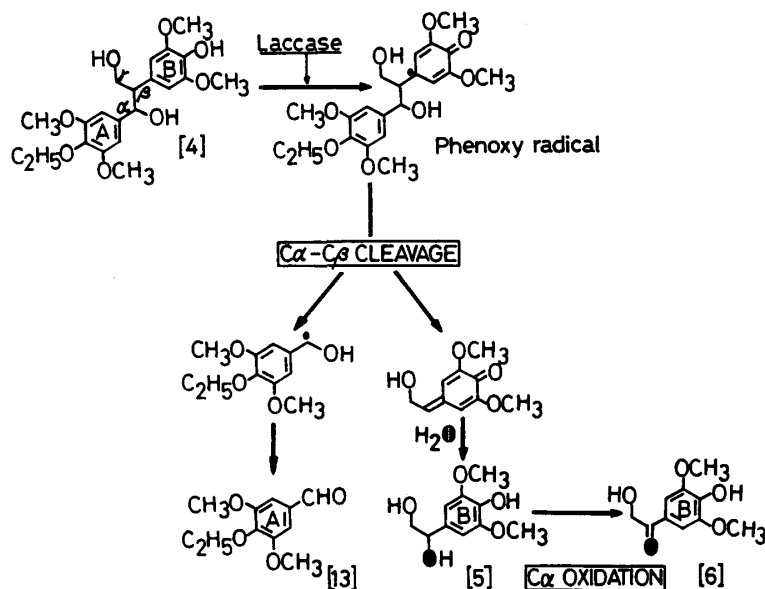


Fig. 17. Degradation pathways of 1-(4-ethoxy-3,5-dimethoxyphenyl)-2-(4-ethoxy-3,5-dimethoxyphenyl)propane-1,3-diol by laccase. ●,  $^{18}\text{O}$ .

## 5. Degradation of diarylpropane-1,3-diol and arylglycerol- $\beta$ -aryl ethers by lignin peroxidase

Lignin peroxidase was isolated from the culture fluid of *P. chrysosporium* as a ligninase to catalyze  $\text{C}_\alpha\text{-C}_\beta$  cleavage of  $\beta$ -1 and  $\beta$ -O-4 model compounds by Tien and Kirk<sup>10)</sup> and Glenn et al.<sup>11)</sup>, respectively. Then, the enzyme was characterized to be a peroxidase whose catalytic cycle is similar to that of other peroxidase<sup>34,35)</sup>: The enzyme catalyzes single electron oxidation of aromatic nuclei to produce cation radicals. These radicals undergo a variety of further reactions. Molecular oxygen was found to react non-enzymatically with the radical intermediates resulting from

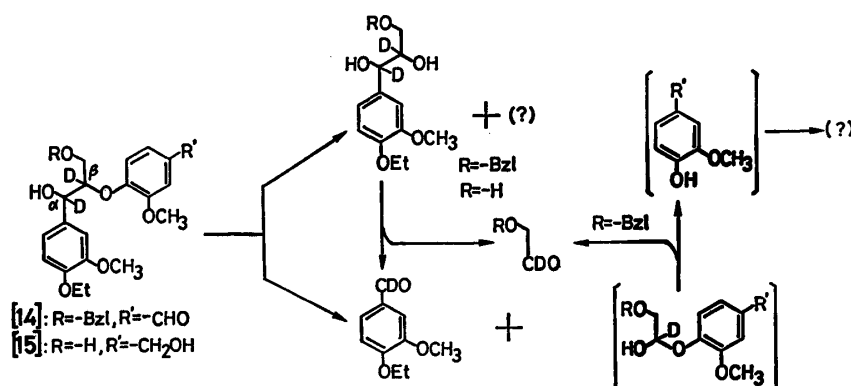


Fig. 18. Degradation pathways of deuterated arylglycerol- $\beta$ -aryl ether models by lignin peroxidase of *P. chrysosporium*. D, Deuterium.

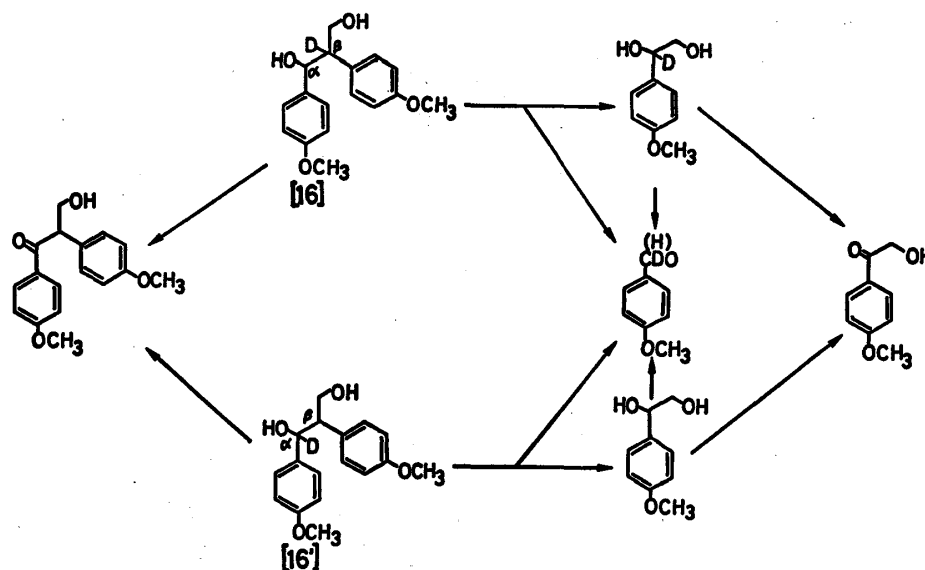


Fig. 19. Degradation pathways of deuterated  $\beta$ -1 models by lignin peroxidase of *P. chrysosporium*. D, Deuterium.

ligninase oxidation.

We used deuterated arylglycerol- $\beta$ -aryl ethers [14, 15] and 1, 2-diarylpropane-1, 3-diols [16, 16'] as substrate for lignin peroxidase isolated from the culture fluid of *P. chrysosporium*. The results<sup>36)</sup> confirmed the formation of previously identified degradation products from both substrates, and showed that the deuterium at  $C_\alpha$  and  $C_\beta$  of the substrates were almost quantitatively retained in the degradation products after the  $C_\alpha$ - $C_\beta$  and ether bond cleavages (Fig. 18 and 19). The results indicated that hydrogen abstraction is not involved in both the  $C_\alpha$ - $C_\beta$  and ether bond cleavages in accordance with the culture experiments with *P. chrysosporium*.

In further investigation<sup>15)</sup> we recently found that a p-benzoquinone monoketal

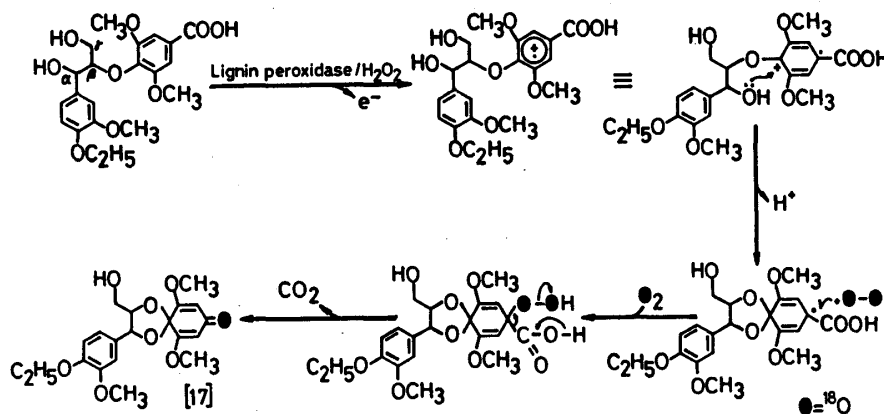


Fig. 20. Formation pathway of p-benzoquinone monoketal from 4-ethoxy-3-methoxyphenylglycerol- $\beta$ -syringic acid ether by lignin peroxidase of *P. chrysosporium*.



[17] is produced in the degradation of 4-ethoxy-3-methoxyphenylglycerol- $\beta$ -syringic acid ether by lignin peroxidase. Isotope experiment with  $^{18}\text{O}_2$  showed that one atom of the oxygen is incorporated into the product from dioxygen. The result is good accordance with the formation of cation radicals of syringyl group by one electron oxidation and that the cation radicals are attacked by  $\alpha$ -hydroxyl group to give cyclohexadienone ketal radicals. The radicals are then attacked to dioxygen to give a peroxide intermediate which is finally decarboxylated to produce p-benzoquinone monoketal (Fig. 20).

### 6. Aromatic ring cleavage of veratryl alcohol and arylglycerol- $\beta$ -aryl ethers by lignin peroxidase

Through extensive and detailed investigations in several laboratories, we now understand a general mechanism for  $\text{C}_\alpha$ - $\text{C}_\beta$  cleavage of the side chain of  $\beta$ -1 and  $\beta$ -O-4 substructure model compounds catalyzed by lignin peroxidase, which involves aryl cation radicals and aryl cation intermediates at the initial enzymatic reaction step.

However, information on aromatic ring cleavage of substructure model compounds by enzymes is deficient, and arylglycerol- $\beta,\gamma$ -cyclic carbonate and arylglycerol- $\beta$ -formate ester have only been identified as aromatic ring cleavage products of  $\beta$ -

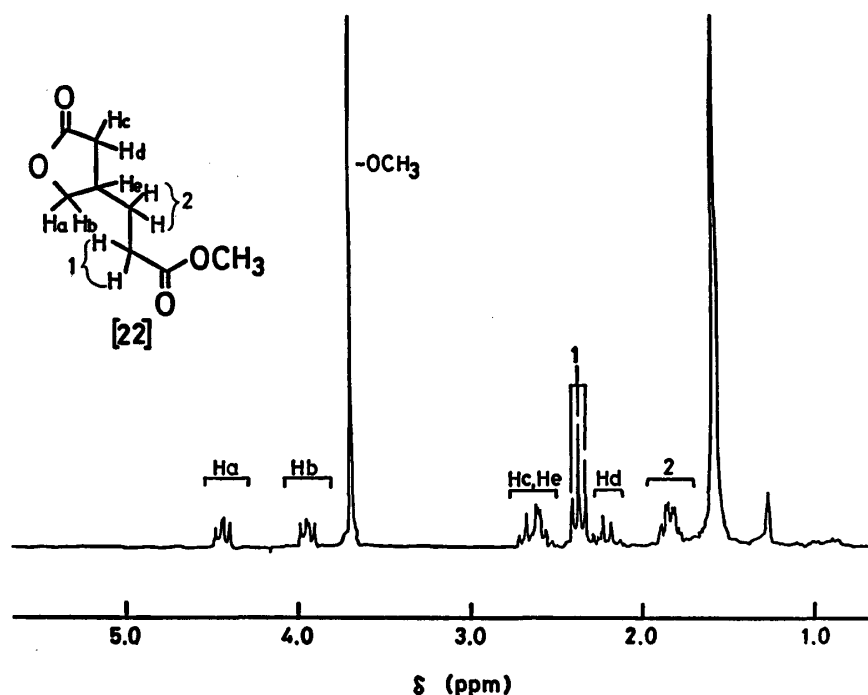


Fig. 21. NMR spectrum of the reduced compound of 5-membered lactones (cis and trans isomers) formed from veratryl alcohol by lignin peroxidase of *P. chrysosporium*.

phenoxy group of arylglycerol- $\beta$ -aryl ether substructure model compounds by ligninolytic cultures of *P. chrysosporium* and *C. versicolor*, respectively.

Recently, Leisola et al.<sup>37)</sup> found that veratryl alcohol [18] was partly converted to 5-membered lactones [20, 21] as ring cleavage products by lignin peroxidase. We<sup>38)</sup> isolated the same compounds in degradation of veratryl alcohol by lignin peroxidase and identified by NMR and mass spectrometry. The ring cleavage products were catalytically reduced by palladium/carbon and the reduced compound [22] was analyzed by NMR and mass spectrometry.

Fig. 21 shows the NMR spectrum of the reduced compound. The NMR spectrum supported the structure of the reduced compound. Fig. 22 shows the mass spectrum of the reduced compound.  $M^+$  peak could not be found by EI (electron impact mass spectrometry) method but  $M^{+1}$  peak was found by CI (chemical ionisation mass spectrometry) of the compound. Thus, molecular weight is 4 mass units higher than the molecular weight of the original compound in agreement with the catalytic reduction of two double bonds of original cleavage products. Both cis- and trans isomers gave the same reduced compound. Fig. 23 shows the degradation pathways of veratryl alcohol [18] to veratryl aldehyde [19] and ring cleavage products [20, 21] (cis- and trans isomers) by lignin peroxidase, and catalytic reduction of the ring cleavage products by palladium/carbon for spectrometric analysis.

In separate experiments<sup>39,40)</sup> we found that  $\beta$ -phenoxy group of arylglycerol- $\beta$ -aryl

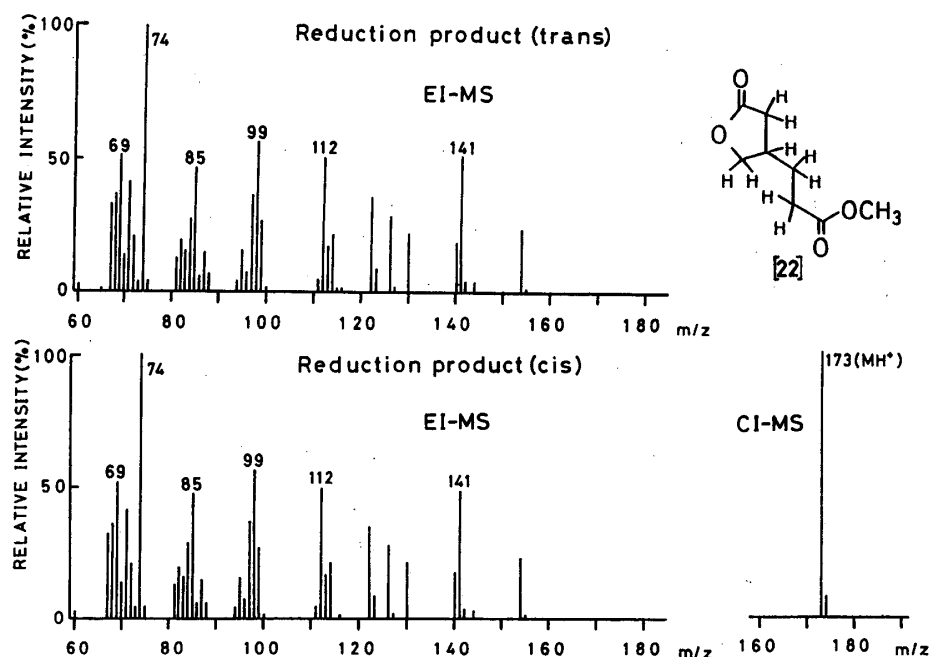


Fig. 22. Mass spectra of the reduced compound of 5-membered lactones (cis and trans isomers) formed from veratryl alcohol by lignin peroxidase of *P. chrysosporium*.

ether compounds are cleaved and converted to arylglycerol- $\beta$ , $\gamma$ -cyclic carbonate [24] and arylglycerol- $\beta$ -formate ester [25] by lignin peroxidase in good agreement with culture experiment. In this experiment we further isolated and identified arylglycerol- $\beta$ -monomethyl oxalate ester [23] as an additional ring cleavage product of arylglycerol- $\beta$ -aryl ether compounds by lignin peroxidase. Fig. 24 shows the mass spectra of

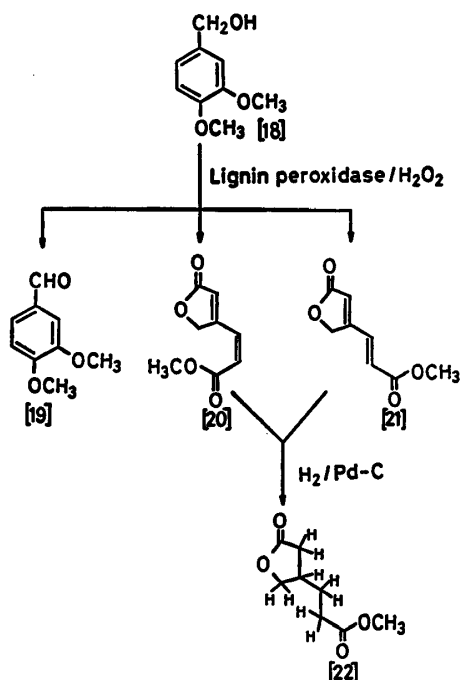


Fig. 23. Degradation pathways of veratryl alcohol to veratryl aldehyde (major product) and aromatic ring cleavage products (cis and trans isomers) by lignin peroxidase of *P. chrysosporium*.

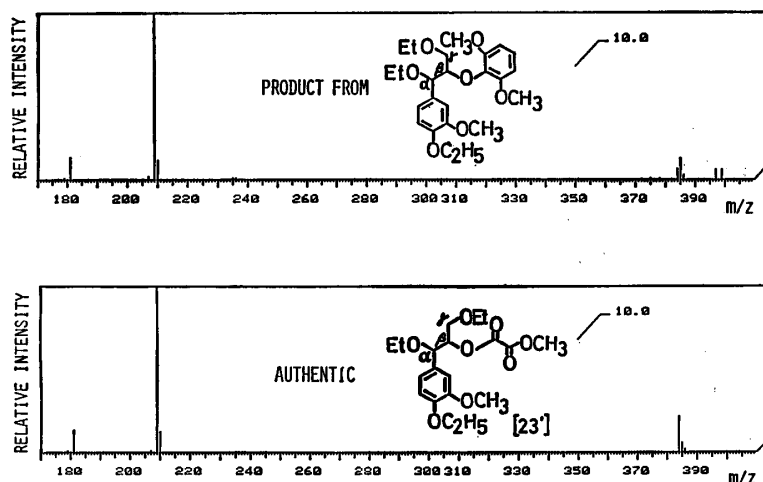


Fig. 24. Mass spectra of arylglycerol- $\beta$ -monomethyl oxalate ester formed from 4-ethoxy-3-methoxyphenylglycerol- $\beta$ -syringyl ether by lignin peroxidase of *P. chrysosporium*.

arylglycerol- $\beta$ -monomethyloxalate ester [23'] isolated as a degradation product of arylglycerol- $\beta$ -syringyl ether and synthesized authentic compound. Molecular ion peak is at 384 and the pattern of fragment ion peaks is identical.

In further experiment<sup>40)</sup> we used arylglycerol- $\beta$ -aryl( $U$ - $^{13}C$ ) ether as substrate and found that the carbon of cyclic carbonate [24] and formate ester [25] of arylglycerol formed by the mediation of lignin peroxidase was derived from  $\beta$ -phenoxy group. C2 carbons and methoxyl group of monomethyloxalate ester [23] were also found to be derived from  $\beta$ -phenoxy group and the methoxyl group of the substrate by the experiment with arylglycerol- $\beta$ -aryl( $U$ - $^{13}C$ ) ether and arylglycerol- $\beta$ -guaiacyl ( $OCD_3$ ) ether as substrate, respectively (Fig. 25). Tracer experiment with  $^{18}O$  further showed that the oxygen of the cyclic carbonate and formate, and one oxygen atom of monomethyloxalate ether were derived from  $H_2^{18}O$ .

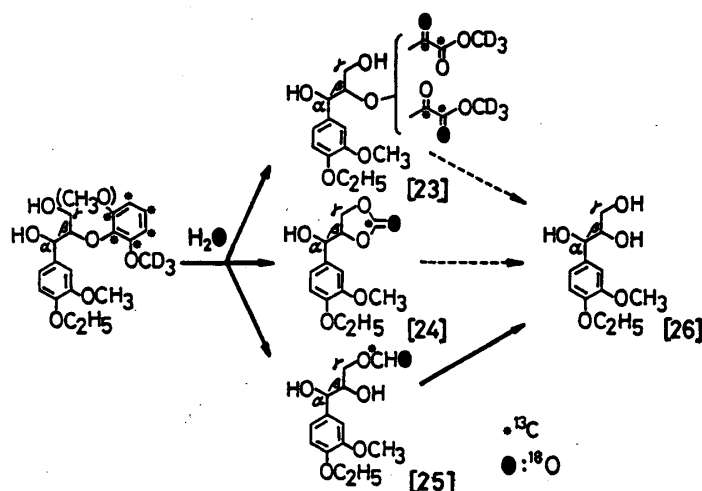


Fig. 25. Formation of arylglycerol- $\beta$ ,  $\gamma$ -cyclic carbonate, formate, and monomethyloxalate esters from arylglycerol- $\beta$ -aryl- $U$ - $^{13}C$ ,  $OCD_3$  ethers by lignin peroxidase of *P. chrysosporium*. D, Deuterium;

Very recently we<sup>40)</sup> found another new product in the degradation of 4-ethoxy-3-methoxyphenylglycerol- $\beta$ -syringyl ether by lignin peroxidase. The product whose peak appeared after substrate on GC column has no acetyltable hydroxyl groups. Molecular ion of the product is 466 which is 32 mass unit higher than that of substrate, indicating the addition of two oxygen atoms to the substrate. Isotope experiment with  $H_2^{18}O$  showed that one of the oxygen atoms of the product was derived from  $H_2^{18}O$ . These results, as shown in Fig. 26, suggest that one of the possible structures of the product is methylarylglyceryl muconate, probably an initial aromatic ring cleavage product which could be converted to monomethyloxalate ester of arylglycerol.

Our recent investigations<sup>39)</sup> further showed that the degradation patterns of

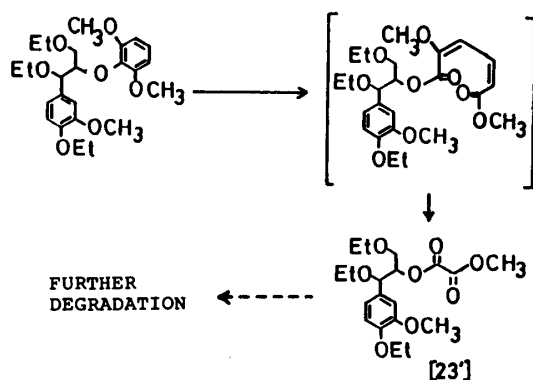


Fig. 26. Methylaryl glyceryl muconate, probably an initial aromatic ring cleavage product in aromatic ring cleavage by lignin peroxidase of *P. chrysosporium*.

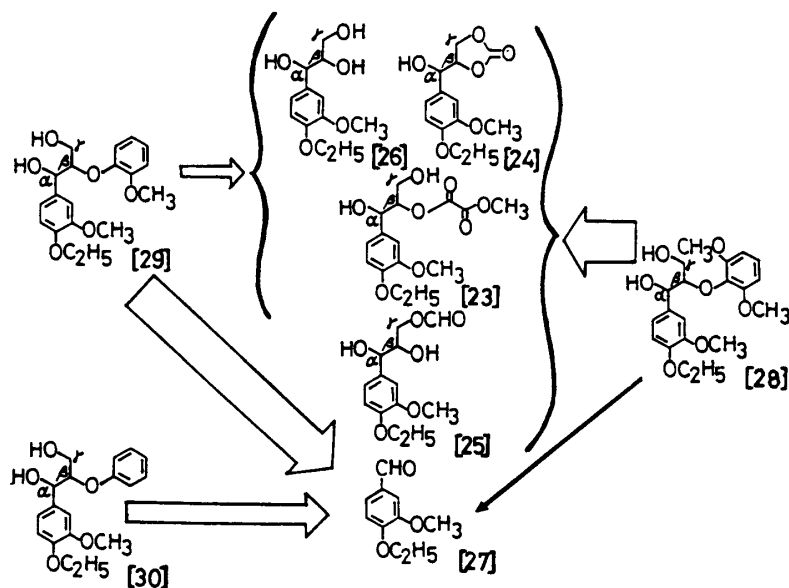


Fig. 27. Degradation patterns of arylglycerol- $\beta$ -aryl ethers with different methoxyl substituents on  $\beta$ -phenoxy groups by lignin peroxidase of *P. chrysosporium*. Broad arrow, Major pathway; narrow solid arrow, minor pathway.

arylglycerol- $\beta$ -aryl ethers by lignin peroxidase (Fig. 27), were remarkably influenced by the methoxyl group on aromatic rings.  $\beta$ -Syringyl ether [28] was mainly degraded to give arylglycerol derivatives [23–27] by aromatic ring cleavage of  $\beta$ -phenoxy group, but the formation of [27] by  $C_\alpha$ - $C_\beta$  cleavage was minor.  $\beta$ -Guaiacyl ether [29] remarkably cleaved between  $C_\alpha$  and  $C_\beta$ , but the aromatic ring cleavage of  $\beta$ -phenoxy group to give arylglycerol derivatives was relatively minor.  $\beta$ -Phenyl ether [30] which has no methoxy group on  $\beta$ -phenoxy group was mostly degraded via  $C_\alpha$ - $C_\beta$  cleavage and no arylglycerol derivatives was detected. These results suggest that the both side chain and aromatic ring cleavages are derived from aryl

cation radicals originally generated by the mediation of lignin peroxidase/ $\text{H}_2\text{O}_2$ , and the contribution for aromatic ring cleavage is remarkably influenced by methoxyl groups on aromatic rings to lead the readiness for the formation of aryl cation radicals.

Although the earlier work<sup>41)</sup> suggested that demethylation of guaiacyl and syringyl groups to give catechol is prerequisite for ring cleavage by dioxygenases, formation of catechol is not always required for lignin peroxidase, which directly catalyzes aromatic ring cleavage of lignin substructure model compounds.

Thus, lignin peroxidase is markedly different from conventional dioxygenases. It is very interesting and noteworthy that the unprecedented aromatic ring cleavage of lignin substructure models does not need conventional dioxygenases, and that lignin peroxidase catalyzes both the cleavage of side chain and aromatic ring of lignin substructure model compounds, although the involvement of other specific enzymes is still anticipated for degradation of monomeric aromatic compounds.

In conclusion, the following unifying view for the role of lignin peroxidase and laccase, and reaction processes in lignin biodegradation is proposed (Fig. 28). Hydrogen peroxide is only required for the conversion of lignin peroxidase into two electron deficient reactive species of compound 1. The compound 1 abstracts stepwise two electrons from aromatic lignin substrates to yield aryl cation radicals or aryl cations, which are attacked by dioxygen or nucleophiles such as water and alcohols, respectively. The subsequent reactions of the cation radicals and cations are not controlled by the enzyme, as in the non-enzyme directed couplings of phenoxy radicals of monolignols in lignin biosynthesis. Cleavage reactions of side chains and aromatic rings are influenced by aromatic ring substitutions and carbonyl groups

### Role of Lignin Peroxidase and Laccase

#### BIOSYNTHESIS

##### Enzymatic reaction

- 1) Peroxidase/ $\text{H}_2\text{O}_2$  ---->  
Phenoxy radicals of monolignols
- 2) Laccase/ $\text{O}_2$  ---->  
Phenoxy radicals of monolignols

##### Non-enzymatic reaction

- 1) Couplings of phenoxy radicals ---->  
Dimeric quinone methides
- 2) Dioxygen attack on carbon-centered radicals
- 3) Nucleophilic attack on quinone methides by  $\text{H}_2\text{O}$  and  $\text{R-OH}$  ---->  
Dilignols

#### BIODEGRADATION

##### Enzymatic reaction

- 1) Lignin peroxidase/ $\text{H}_2\text{O}_2$  ---->  
Aryl cation radicals or cations of non-phenolic units
- 2) Laccase/ $\text{O}_2$  ---->  
Phenoxy radicals of phenolic units

##### Non-enzymatic reaction

- 1) Homolytic or heterolytic cleavage of side chains ( $\text{C}_\alpha$ - $\text{C}_\beta$ , alkyl-phenyl) and aromatic rings
- 2) Dioxygen attack on carbon-centered radical intermediates
- 3) Nucleophilic attack on aryl cations and  $\text{C}_\alpha$  cations by  $\text{H}_2\text{O}$  and  $\text{R-OH}$  ---->  
Degradation products

Fig. 28. Role of lignin peroxidase and laccase in biosynthesis and biodegradation of lignin.

at the side chain. Rate of ring cleavage of syringyl group of arylglycerol- $\beta$ -syringyl ether is much higher than those of guaiacyl and p-hydroxyphenyl groups by electron donating effect of  $\text{OCH}_3$ .  $\text{C}_\alpha\text{-C}_\beta$  cleavage is retarded by  $\text{C}_\alpha$  carbonyl group of side chain by electron withdrawing effect. The radicals and cation intermediates in the cleavage of side chains and aromatic rings are attacked by  $\text{O}_2$ ,  $\text{H}_2\text{O}$  and  $\text{R-OH}$  non-enzymatically, probably depending on the reaction conditions, such as  $\text{O}_2$  pressure, pH of the reaction medium etc. Thus the oxygen species from either dioxygen or water introduced into the degradation products are not controlled by the enzyme, which is sharp contrast to the reaction process mediated by conventional dioxygenases. The degradation mechanism of phenolic substructure models mediated by laccase such as  $\text{C}_\alpha\text{-C}_\beta$  cleavage, alkyl-phenyl cleavage,  $\text{C}_\alpha$  oxidation and probably aromatic ring cleavage is mostly similar, except that the laccase can oxidize phenolic compounds to their phenoxy radicals but the lignin peroxidase both phenolic and non-phenolic compounds.

Fig. 29 shows the degradation products identified so far from  $\beta\text{-O-4}$  substructure model compounds by culture of *P. chrysosporium* and lignin peroxidase. These are yielded by  $\text{C}_\alpha\text{-C}_\beta$  cleavage, O-4 cleavage, alkyl-phenyl cleavage,  $\text{C}_\alpha$  oxidation, oxidation of  $\beta$ -phenoxy group and subsequent reaction with hydroxyl groups, and aromatic ring cleavage derived from the oxidation of either A ring or B ring by lignin peroxidase.

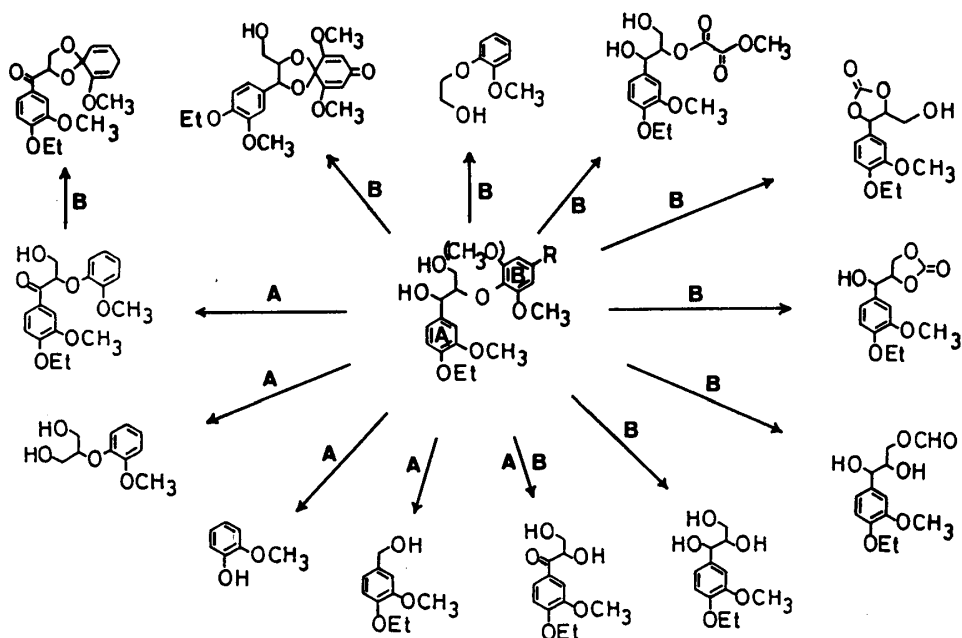


Fig. 29. Degradation products identified from  $\beta\text{-O-4}$  substructure model compounds by the culture of *P. chrysosporium* and its lignin peroxidase. A, Degradation pathway started from aromatic ring A; B, degradation pathway started from aromatic ring B.

Lignin is a complex aromatic polymer linked by various ether- and carbon to carbon linkages and contains 10–20% phenolic hydroxyl groups which are attacked by both lignin peroxidase and laccase. The phenolic group would be increased during lignin depolymerization by C<sub>α</sub>-C<sub>β</sub> cleavage of nonphenolic β-O-4 substructures and subsequent hydrolysis of the resulting ketals to give phenolic hydroxyl groups. Thus, participation of both lignin peroxidase and laccase in lignin depolymerization and aromatic ring cleavage as ligninases is highly probable.

### Acknowledgment

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### References

- 1) T. HIGUCHI: Biosynthesis and Biodegradation of Wood Components, T. HIGUCHI (ed.), Academic Press Inc., Orlando, FL, pp. 557–578 (1985).
- 2) T. HIGUCHI and F. NAKATSUBO: *Kemia-Kemi*, **9**, 481 (1980).
- 3) S. IWAHARA, M. KUWAHARA and T. HIGUCHI: *Hakkokogaku*, **55**, 325 (1977).
- 4) T.K. KIRK, E. SCHULTZ, W.J. CONNORS, L.F. LORENZ and J.G. ZEIKUS: *Arch. Microbiol.*, **117**, 277 (1978).
- 5) T. HIGUCHI: *Wood Res. (Kyoto)*, **67**, 47 (1981).
- 6) T. UMEZAWA, F. NAKATSUBO and T. HIGUCHI: *Arch. Microbiol.*, **131**, 124 (1982).
- 7) Y. KAMAYA and T. HIGUCHI: *Mokuzai Gakkaishi*, **29**, 789 (1983).
- 8) Y. KAMAYA and T. HIGUCHI: *Mokuzai Gakkaishi*, **30**, 237 (1984).
- 9) T. UMEZAWA, F. NAKATSUBO and T. HIGUCHI: *Agric. Biol. Chem.*, **47**, 2677 (1983).
- 10) M. TIEN and T.K. KIRK: *Science*, **221**, 661 (1983).
- 11) J.K. GLENN, M.A. MORGAN, M.B. MAYFIELD, M. KUWAHARA and M.H. GOLD: *Biochem. Biophys. Res. Commun.*, **114**, 1077 (1983).
- 12) A. ENOKI, G.P. GOLDSBY and M.H. GOLD: *Arch. Microbiol.*, **125**, 227 (1980).
- 13) T. UMEZAWA and T. HIGUCHI: *FEBS Lett.*, **192**, 147 (1985).
- 14) K. MIKI, V. RENGANATHAN and M.H. GOLD: *Proceed. The 3rd Intern. Conf. Biotechnol. Pulp Paper Ind.*, Stockholm, pp. 13–16 (1986).
- 15) S. KAWAI, T. UMEZAWA and T. HIGUCHI: *FEBS Lett.*, in press.
- 16) H. ISHIKAWA and T. OKI: *Mokuzai Gakkaishi*, **10**, 207 (1964).
- 17) T. FUKUZUMI, H. TAKATSUKA and K. MINAMI: *Arch. Biochem. Biophys.*, **129**, 396 (1969).
- 18) T. UMEZAWA and T. HIGUCHI: *Wood Res. (Kyoto)*, **71**, 25 (1985).
- 19) T. UMEZAWA, T. HIGUCHI and F. NAKATSUBO: *Agric. Biol. Chem.*, **47**, 2945 (1983).
- 20) T. UMEZAWA and T. HIGUCHI: *Agric. Biol. Chem.*, **48**, 1917 (1984).
- 21) T. UMEZAWA and T. HIGUCHI: *FEMS Microbiol. Lett.*, **26**, 123 (1985).
- 22) T. UMEZAWA and T. HIGUCHI: *FEBS Lett.*, **182**, 257 (1985).
- 23) S. KAWAI, T. UMEZAWA and T. HIGUCHI: *App. Environ. Microbiol.*, **50**, 1505 (1985).
- 24) F. NAKATSUBO, I.D. REID and T.K. KIRK: *Biochem. Biophys. Acta*, **719**, 284 (1982).
- 25) A. ENOKI and M.H. GOLD: *Arch. Microbiol.*, **132**, 123 (1982).
- 26) T.K. KIRK and F. NAKATSUBO: *Biochem. Biophys. Acta*, **756**, 376 (1983).



- 27) Y. KAMAYA and T. HIGUCHI: FEMS Microbiol. Lett., **22**, 89 (1984).
- 28) M. TIEN and T.K. KIRK: Proc. Natl. Acad. Sci. USA, **81**, 2280 (1984).
- 29) M.H. GOLD, M. KUWAHARA, A.A. CHIU and J.K. GLENN: Arch. Biochem. Biophys., **234**, 353 (1984).
- 30) F. NAKATSUBO, T.K. KIRK, M. SHIMADA and T. HIGUCHI: Arch. Microbiol., **128**, 416 (1981).
- 31) P. ANDER, A. HATAKKA and K.-E. ERIKSSON: Microbiology, Chemistry and Potential Applications, T.K. KIRK, T. HIGUCHI and H.-m. CHANG eds., Vol. 2, pp. 1-16, CRC Press.
- 32) T. UMEZAWA, T. HIGUCHI and F. NAKATSUBO: Abst. 32th Ann. Meeting Wood Res. Soc., p. 234, Fukuoka, Japan (1982).
- 33) S. KAWAI, T. UMEZAWA, M. SHIMADA and T. HIGUCHI: Proceed. 3rd Intern. Conf. Biotechnol. Pulp Paper Ind., Stockholm, June 16-19, 1986, p. 39.
- 34) V. RENGANATHAN and M.H. GOLD: Biochemistry, **25**, 1626 (1986).
- 35) M. TIEN, T.K. KIRK, C. BULL and J.A. FRE: J. Biol. Chem., **261**, 1687 (1986).
- 36) T. HABE, M. SHIMADA, T. UMEZAWA and T. HIGUCHI: Agric. Biol. Chem., **49**, 3505 (1985).
- 37) M.S.A. LEISOLA, B. SCHMIDT, U.T. WYSS and A. FIECHTER: FEBS Lett., **189**, 267 (1985).
- 38) T. HATTORI, M. SHIMADA, T. UMEZAWA and T. HIGUCHI: Manuscript in preparation.
- 39) T. UMEZAWA, M. SHIMADA, T. HIGUCHI and K. KUSAI: FEBS Lett., **205**, 287 (1986).
- 40) T. UMEZAWA and T. HIGUCHI: FEBS Lett., **205**, 293 (1986).
- 41) R.B. CAIN: Lignin Biodegradation: Microbiology, Chemistry and Potential Applications, T.K. KIRK, T. HIGUCHI and H.-m. CHANG eds., Vol. 1, pp. 21-60, CRC Press, Boca Raton, FL.